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Emodin ameliorates glucose-induced matrix synthesis in human peritoneal mesothelial cells

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Background. Prolonged exposure of human peritoneal mesothelial cells (HPMC) to high glucose concentrations in peritoneal dialysate is the principal factor leading to matrix accumulation and thickening of the peritoneal membrane, accompanied by progressive deterioration of transport functions. These changes are mediated in part through protein kinase C (PKC) activation and the induction of transforming growth factor-beta 1 (TGF-β1). Emodin (3-methyl-1,6,8 trihydroxyanthraquinone) has previously been demonstrated to reduce cell proliferation and fibronectin synthesis in cultured mesangial cells. How emodin modulates glucose-induced abnormalities in HPMC has not been elucidated and thus constitutes the theme of this study.

Methods. We investigated the effects of emodin on the expression of PKCα, TGF-β1, fibronectin, and collagen type I in HPMC, and its effects on HPMC proliferation under physiologic (5 mmol) or high (30 mmol) glucose concentrations.

Results. Exposure of HPMC cultured with 5 mmol or 30 mmol D-glucose to emodin (20 μg/mL) resulted in an initial lag of proliferation by 2.3 to 2.7 days, but did not affect cell viability or morphology at confluence. D-glucose (30 mmol) induced TGF-β1 secretion in a time-dependent manner (3.72 ± 0.29 and 4.30 ± 0.50 pg/μg cellular protein at 24 hours and 48 hours respectively, compared to 2.13 ± 0.23 and 2.65 ± 0.32 pg/μg cellular protein at 24 hours and 48 hours, respectively for 5 mmol glucose; $P < 0.001$ at both time points). Such induction was ameliorated by emodin (20 μg/mL) (TGF-β1 concentration 2.25 ± 0.15 and 2.96 ± 0.33 pg/μg cellular protein at 24 hours and 48 hours, respectively, in the presence of emodin and 30 mmol D-glucose; $P < 0.001$ compared to 30 mmol D-glucose alone at both time points). Induction of TGF-β1 synthesis by 30 mmol D-glucose was associated with induction of PKCα, phosphorylation of cAMP-responsive element binding protein (CREB) and activating transcription factor-1 (ATF-1), and increased fibronectin and type I collagen translation. Emodin abrogated all these effects of concentrated glucose. Immunohistochemical staining showed that 30 mmol D-glucose induced cytoplasmic, perinuclear, and extracellular fibronectin and type I collagen expression by HPMC. Emodin reduced 30 mmol D-glucose-induced cytoplasmic and extracellular matrix synthesis to near basal levels.

Conclusion. Our findings demonstrate that emodin ameliorates the undesirable effects of concentrated glucose on HPMC via suppression of PKC activation and CREB phosphorylation, and suggest that emodin may have a therapeutic potential in the prevention or treatment of glucose-induced structural and functional abnormalities in the peritoneal membrane.

The peritoneal membrane provides a protective anatomical barrier and a frictionless interface for the movement of organs and tissues in the peritoneal cavity. Its constituents include an uppermost protective monolayer of mesothelial cells, a basal lamina, and the submesothelium, which contains capillaries, fibroblasts, and collagenous fibrous tissue [1]. Peritoneal thickening and fibrosis can result from bacterial infection, surgery, or chemical or metabolic toxicity, either indirectly through the induction of pro-inflammatory cytokines and growth factors, or directly through modulations in the synthesis and/or degradation of extracellular matrix [2–4]. Mesothelial cells play a critical role in the synthesis and remodeling of the extracellular matrix and contribute to the synthesis of cytokines and growth factors within the peritoneal cavity [3, 5–7]. Injury to mesothelial cells represents an important step in the initiation of pathologic changes in the peritoneal membrane.

Peritoneal dialysis (PD) is an important treatment for patients with end-stage renal failure. Conventional peritoneal dialysates are not physiologic, and they utilize glucose at high concentrations to provide the osmotic drive for the removal of excessive fluid from the body. Continuous prolonged exposure of the peritoneal membrane to peritoneal dialysate leads to chronic activation of the mesothelium, with increased synthesis and secretion of pro-inflammatory mediators and increased deposition of

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matrix proteins [8, 9]. These processes are further exacerbated during episodes of peritonitis. The peritoneum thus undergoes progressive structural and functional deterioration, leading ultimately to failure of PD [10–12].

We, and others, have investigated the role of mesothelial cells in the pathogenesis of peritoneal fibrosis. In vitro studies have demonstrated that high glucose concentrations can modulate cell proliferation and up-regulate matrix protein synthesis in peritoneal mesothelial cells. These changes are mediated by protein kinase C (PKC) activation and the induction of transforming growth factor-beta 1 (TGF-β1) synthesis [13–15]. PKC is a serine-threonine kinase that plays a key role in intracellular signal transduction of hormones and cytokines [16]. It has at least 12 isoforms, and it remains unclear which isoforms are involved in the induction of matrix synthesis and TGF-β1 in peritoneal mesothelial cells during long-term PD. Given that TGF-β1 induction is similarly observed in diabetes mellitus, we postulate that PKCα, an isoform well documented to be activated in diabetic nephropathy [17], may also play a pivotal role in glucose-induced TGF-β1 expression in peritoneal mesothelial cells.

While TGF-β1 is vital to normal tissue repair, overexpression of TGF-β1 has been strongly implicated as a major pathogenetic mechanism leading to tissue fibrosis in different diseases [3, 18]. Previous attempts to alleviate TGF-β1–mediated fibrosis have included the intraperitoneal administration of glycosaminoglycans, systemic administration of anti-TGF-β, or blockade of angiotensin II by angiotensin-converting enzyme (ACE) inhibitors [19–22]. Emodin (3-methyl-1,6,8 trihydroxyanthraquinone), a natural anthraquinone extracted from *Rheum*, has been demonstrated to reduce fibronectin (FN) synthesis in TGF-β1–stimulated mesangial cells, and reduce renal hypertrophy in rats with streptozotocin-induced diabetic kidney disease [23, 24]. In addition, emodin inhibits tyrosine kinase activity and possesses anti-inflammatory and anti-proliferative properties [25–29]. In this context, studies have shown that emodin could reduce protein-tyrosine phosphorylation in bronchial epithelial cells, inhibit NF-kappaB (NFκB) activation in endothelial cells, reduce proliferation and cytokine synthesis in mesangial cells and activated T cells, and initiate bacterial DNA damage [25–28]. Given the pivotal role of mesothelial cells in peritoneal inflammation and fibrosis during PD, and their constant exposure to the high dialysate glucose concentration, we have been investigating the effects of high glucose concentration on human peritoneal mesothelial cells (HPMC) and potential modulatory mechanisms. We have reported that emodin ameliorated the morphologic changes and chronic FN synthesis in HPMC induced by 30 mmol D-glucose [30]. The present study examined the effects of concentrated glucose on HPMC proliferation and TGF-β1, as well as matrix [FN and collagen type I (Coll-I)] synthesis by HPMC. We also investigated the possible role of PKCα in mediating the profibrotic processes, and the mechanisms by which emodin modulated these glucose-induced changes. Since elevated glucose concentrations and TGF-β1 have been shown to regulate FN transcription in human mesangial cells through a consensus cAMP response element (CRE) located at –170 bp of the FN gene [31, 32], and PKC can activate CRE-binding protein (CREB) [32], we also investigated CREB phosphorylation in HPMC under high glucose concentration in the presence or absence of emodin. Our results showed that emodin ameliorated glucose-induced TGF-β1 and matrix synthesis in HPMC by inhibiting PKCα activation and phosphorylation of CREB.

METHODS

Materials

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated and were of the highest purity. Tissue culture flasks were purchased from Falcon (Becton Dickinson, Hong Kong) and culture media and supplements from Invitrogen Hong Kong Ltd. (Hong Kong, China). Antisera to human FN (clone IST-4), Coll-I (clone COL-1 or C-18), TGF-β1 (sc-146), PKCα (clone MC5), and actin (sc-8432) were purchased from Sigma Chemical Co. and TWC BioSearch International (Santa Cruz, CA, USA). The PhosphoPlus CREB (Ser133) antibody kit (New England BioLabs, Beijing, China; TWC Biosearch International) was used to detect CREB and phosphoCREB. Tyrosine kinase assay kit was purchased from Sigma Chemical Co. Cytotoxicity detection kit [lactate dehydrogenase (LDH)] was purchased from Boehringer Mannheim (Roche Diagnostics GmbH, Mannheim, Germany). [^{3}H]-thymidine (methyl-[^{3}H-thymidine) was purchased from Amersham Biosciences China Limited (Hong Kong, China). Emodin was extracted from *Rheum officinale* and fraction-distilled to above 98% purity, and characterized by high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and mass spectrometry [33]. Endotoxin-free emodin was dissolved in 0.1 mol/L NaOH at a stock concentration of 4 mg/mL, and diluted in Medium 199 prior to use in experiments. Human umbilical vascular endothelial cells (HUVEC) and normal human bronchial epithelial cells (NHBE) were purchased from Clonetics (San Diego, CA, USA), and human pleural mesothelial cells (MeT-5A) and mink lung epithelial cells (MLEC; CCL-64) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA).

Cytochemical analysis of peritoneal biopsies

Parietal peritoneal biopsies were obtained from 5 patients at the time of initial insertion of the PD catheter,

and from 8 patients who had been on PD for more than 12 months after informed consent, at the time of removal, or reinsertion of the peritoneal dialysis catheter. Paraffin sections (10 μm) were incubated with primary antibodies against human FN, Coll-I, or TGF-β1 (1:50 for all antibodies) overnight at room temperature, and incubated with relevant secondary antibodies conjugated to horseradish peroxidase for 1 hour at 37°C. Signal detection and visualization was achieved using the peroxidase-antiperoxidase (PAP) method (Dako; Gene Company, Ltd., Hong Kong, China), counterstained with hematoxylin, and specimens examined using a Leica DMLB microscope (Leica Microsystems, Ltd., Hong Kong, China). Specimens were photographed with Kodak Max 100 film using a MPS60 camera.

Culture of cells

HPMC were obtained by enzymatic digestion of omental specimens from nonuremic patients who underwent abdominal surgery, and cultured in Medium199 supplemented with 10% fetal calf serum (FCS) as previously described [5, 6]. All experiments were performed on cells of the second passage that had been growth arrested for 72 hours. After synchronization of the cell cycle, HPMC were cultured under physiologic D-glucose concentration (5 mmol), or elevated D-glucose concentration (30 mmol) for periods up to 1 week, or exposed to phorbol 12-myristate 13-acetate (PMA) (100 nmol) for up to 48 hours, in the presence or absence of emodin (20 μg/mL). D-glucose (30 mmol) represented the intraperitoneal glucose concentration after 1 hour of equilibration following the instillation of conventional peritoneal dialysate, which contained glucose at 75 mmol or 120 mmol. Mannitol or L-glucose was used as hexose control in parallel experiments, at concentrations identical to those of D-glucose. In separate experiments, TGF-β1 neutralizing antibody (1 μg/mL) was added to HPMC 1 hour prior to stimulation by elevated glucose or PMA. Human peritoneal fibroblasts (HPF) were cultured according to the method of Beavis et al [34], and human mesangial cells (HMC), glomerular epithelial cells (GEC), and proximal tubular epithelial cells (PTEC) by differential sieving of nephrectomized renal specimens [35–37].

Assessment of cell proliferation

[^{3}H]-thymidine incorporation. Cells were seeded into 96-well plates at a density of 10,000/cm^2 and cultured in Medium 199 containing 10% FCS until 80% confluent. Cells were washed twice with PBS to remove unattached cells and further incubated with Medium 199 containing either 5 mmol or 30 mmol D-glucose and [^{3}H]-thymidine (1 μCi/mL) in the presence or absence of emodin (0 to 200 μg/mL) for 24 hours. At the end of the time point, the supernatant was decanted, cells trypsinized with 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA), incorporated radioactivity precipitated with 10% trichloroacetic acid (TCA) (100 μL), washed twice with TCA, and dissolved with 0.1 mol NaOH (200 μL). [^{3}H]-thymidine incorporation was detected by beta liquid scintillation counting.

Direct cell counting. Cells were seeded into 24-well plates at a density of 10,000/cm^2 and cultured for 24 hours in Medium 199 containing 10% FCS. Cells were washed twice with PBS to remove unattached cells and further incubated with Medium 199 containing either 5 mmol or 30 mmol D-glucose in the presence or absence of emodin for periods up to 7 days. At selective time periods, HPMC were trypsinized as described above, and counted using a Neubauer chamber (Sigma Chemical Co.).

Determination of lactate dehydrogenase (LDH) release

Confluent HPMC were cultured under control and experimental conditions in 96-well tissue culture plates for 24 hours. Supernatants were collected, centrifuged for 10 minutes at 2000*g*, and assessed for LDH release using a commercially available cytotoxicity kit according to the manufacturer's instructions. Cytotoxicity of control and experimental samples were expressed as the percentage of LDH release compared to total intracellular LDH content, the latter determined by lysis of representative cell monolayers using 2% Triton X-100 (vol/vol) according to the manufacturer's instructions.

Measurement of cellular protein concentration

HPMC cultured in triplicate in 96-well plates under control or experimental conditions were lysed with 4 mol urea buffer, 20 mmol sodium acetate, pH 6.0 containing 1% (vol/vol) Triton X-100 (50 μL). Protein concentration in cell lysate was determined using a modified Lowry assay according to the manufacturer's instructions (Bio-Rad Pacific, Ltd., Hong Kong, China).

Determination of TGF-β1 concentration in culture supernatant

Samples (1 mL) of culture supernatant were lyophilized, reconstituted to 200 μL in PBS containing 1% fatty acid free bovine serum albumin (BSA), and assayed using a commercial TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Onwon Trading, Ltd., Hong Kong). Cross-reactivity with other TGF-β isoforms was less than 2%.

Determination of TGF-β1 bioactivity

The mink lung epithelial cell (MLEC) bioassay for TGF-β1 was performed as described by Ha and colleagues [13] with slight modification. Briefly, MLEC (passage 50–55) were subcultured into 96-well culture plates at 10,000 cells/cm^2 and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS for 2 to 4 hours. MLEC were then exposed to serially

diluted recombinant human TGF-β1 (0 to 1000 pg/mL) or spent culture media from HPMC obtained under control and experimental conditions for 72 hours at 37°C. Thereafter, [^{3}H]-thymidine (1 μCi/mL) was added to each well and the incubation continued for another 24 hours. At the end of the incubation period, cells were washed three times with ice-cold PBS, trypsinized with 0.05% trypsin/0.02% EDTA, incorporated radioactivity precipitated with 10% TCA (100 μL), washed twice with TCA, and dissolved with 0.1 mol NaOH (200 μL). [^{3}H]-thymidine incorporation was detected by beta liquid scintillation counting. A standard curve was constructed from the varying degrees of inhibition on MLEC growth observed with corresponding concentrations of recombinant human TGF-β1.

Determination of tyrosine kinase activity

Confluent HPMC cultured in 35 mm culture dishes under control and experimental conditions were washed twice with cold PBS and lysed at 4°C with 50 mmol N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer pH 7.4, containing 1% Triton X-100, 10% glycerol, 1 mmol dithiothreitol, 1 mmol activated sodium vanadate (100 μg/mL) in the presence of proteinase inhibitors (final concentration 1 mmol benzamidine, 10 μg/mL leupeptin, and 2 μg/mL pepstatin A). Cell lysates were centrifuged at 10,000*g* for 15 minutes at 4°C to remove cell debris, and assayed for tyrosine kinase according to the manufacturer's instructions (Sigma Chemical Co.). Tyrosine kinase activity is often associated with receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR). Thus, EGFR (4U) was used as the positive control and incubated with emodin (20 μg/mL) on ice for 10 minutes prior to assay.

Determination of FN and Coll-I synthesis by HPMC using enzyme-linked immunosorbent assay

HPMC were cultured in duplicate in 35 mm culture dishes under control or experimental conditions for 48 hours. The supernatants were decanted and cells lysed with 4 mol urea buffer, 20 mmol sodium acetate, pH 6.0 containing 1% (vol/vol) Triton X-100 (100 μL). Cell lysates were desalted, buffer-exchanged into PBS using Ultrafree Centrifugal Concentrators (Millipore China Ltd., Hong Kong, China), and assayed for FN and Coll-I using "in-house" ELISAs [38]. Briefly, 96-well microtiter plates (Immunlon 2; Dynex Technologies, Inc., Chantilly, VA, USA) were coated in duplicate with FN or Coll-I (0 to 500 ng/mL) in serial dilutions, or HPMC supernatant (starting dilution 1:10) or cell lyaste (starting dilution 1:10) in 15 mmol Na_2CO_3 and 35 mmol $NaHCO_3$, pH 9.6 overnight at 4°C. Plates were washed three times with PBS/0.1% Tween-20 in between all steps. The plates were blocked with 3% bovine serum albumin for 1 hour at 37°C and incubated with primary antibodies (1:1500 for mouse anti-human FN and 1:1000 for goat anti-human Coll-I) for 1 hour at 37°C. The relevant horseradish peroxidase–conjugated secondary antibody was then added for 1 hour at 37°C. Bound immunoglobulins were detected by the addition of Sigma Fast™ (Sigma Chemical Co., St. Louis, MO, USA) o-phenylenediamine dihydrochloride tablets dissolved according to the manufacturer's instructions, and the absorbance read at a wavelength of 450 nm. Prior experiments demonstrated <5% cross-reactivity of primary antibodies with other matrix proteins. Intra-assay and interassay coefficients of variance were 4.08 ± 1.5% and 5.7 ± 2.7%, respectively, for FN, and 6.48 ± 2.9% and 6.95 ± 3.4%, respectively, for Coll-I.

Preparation of cytosolic and membrane extracts

Separation of cytosolic and membrane proteins was achieved as described by Ma and colleagues [39] with slight modifications. Briefly, confluent HPMC exposed to control and experimental conditions for time periods up to 48 hours were scraped into PBS using a rubber policeman and collected by centrifugation at 1500*g* for 10 minutes. Cell pellets were sonicated five times for 10 seconds each period in 200 μL PBS containing protease inhibitors [6], and fractionated by ultracentrifugation at 100,000*g* for 30 minutes at 4°C. Cytosolic proteins appeared in the supernatant, while pelleted membrane proteins were resuspended in 20 mmol sodium acetate, pH 6.0, containing 4 mol urea and 1% Triton X-100 (100 μL). The protein concentration of each sample was determined by a modified Lowry assay (Bio-Rad Pacific, Ltd.) prior to Western blot analysis.

Preparation of nuclear extract

Confluent HPMC exposed to control and experimental conditions for 48 hours were scraped into PBS using a rubber policeman and collected by centrifugation at 1500*g* for 10 minutes as previously described. Nuclear extracts were prepared as described by Abmayr and Workman [40] with slight modifications. Briefly, the cell pellet was resuspended in 10 mmol HEPES (pH 7.9) containing 1.5 mmol $MgCl_2$, 10 mmol KCl, 0.5 mmol dithiothreitol, and 0.2 mmol phenylmethylsulfonyl fluoride, and homogenized with a tissue tearor (BioSpec Products, Inc., Bartlesville, OK, USA) at 12,000 rpm for 2 minutes. Release of nuclei was monitored under phase contrast microscopy. Isolated nuclei were separated from cytoplasmic contents by centrifugation at 3300*g* for 15 minutes and nuclear proteins extracted with 20 mmol HEPES (pH 7.9) containing 25% (vol/vol) glycerol, 1.5 mmol $MgCl_2$, 0.8 mmol KCl, 0.2 mmol EDTA, 0.5 mmol dithiothreitol, and 0.2 mmol phenylmethylsulfonyl fluoride. The samples were then dialyzed against 20 mmol HEPES buffer (pH 7.9) containing 20% (vol/vol) glycerol, 100 mmol KCl, 0.5 mmol dithiothreitol, and the aforementioned protease inhibitors. The protein concentration of

each sample was determined by a modified Lowry assay (Bio-Rad Pacific, Ltd.) prior to Western blot analysis.

Western blot analyses—Cytosolic and membrane fractions for PKCα activation, total cell lysate for matrix protein expression, and nuclear extract for CREB phosphorylation

Whole cell lysates were obtained by solubilizing HPMC in 4 mol of urea buffer (500 μL). Whole cell lysates, cytosolic, membrane, or nuclear extracts (10 μg total protein content) was denatured in sample buffer at 95°C for 5 minutes and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were electrophoresed on 7.5% and 10% acrylamide gels, respectively, to investigate FN and Coll-I synthesis, or 12% gels to investigate translation of PKCα and CREB. Proteins were transferred onto nitrocellulose membrane using a mini-gel transfer system (Bio-Rad Pacific, Ltd.) at 100 V for 1 hour at 4°C. Equal loading of proteins was confirmed by staining of membranes with 1% (weight/vol) Ponceau S solution. Membranes were immunoblotted as previously described [35] using respective primary (1:750 for antibodies to PKCα or FN; 1:1000 for antibodies to Coll-I, total CREB, or phosphoCREB) and secondary antibodies. The bands were visualized by enhanced chemiluminescence (ECL), semiquantitated by densitometry using ChemiGenius analysis software (Syngene, Ltd., Cambridge, UK) and unless otherwise stated expressed as arbitrary densitometric unit (DU). Expression of PKCα, FN, and Coll-I was normalized to actin synthesis, and phosphoCREB was normalized to total CREB.

Immunohistochemical staining of HPMC

Control and stimulated HPMC were fixed with cold acetone/methanol (1:1) for 5 minutes prior to washing with PBS [41]. Cells were blocked with 3% BSA in PBS for 1 hour at room temperature followed by washing with PBS for 30 minutes. Cells were then incubated with primary antibody (1:50 for antibody to PKCα, TGF-β1, or Coll-I; 1:100 for antibody to FN) for 1 hour at 37°C in a humidified chamber. Cells were subsequently washed with PBS and the relevant fluoroisothiocyanate (FITC)-conjugated secondary antibody applied for 1 hour at 37°C in a darkened humidified chamber. After washing with PBS the cells were mounted with fluorescence mountant (Dako; Gene Company), and epifluorescence was viewed using an Axiovert Zeiss 135 inverted microscope.

Statistical analysis

Results are expressed as mean ± SD. Unless otherwise stated, all experiments were performed four times using HPMC from four separate donors. Data from experimental groups and controls were compared by the Student *t* test. $P < 0.05$ was considered statistically significant.

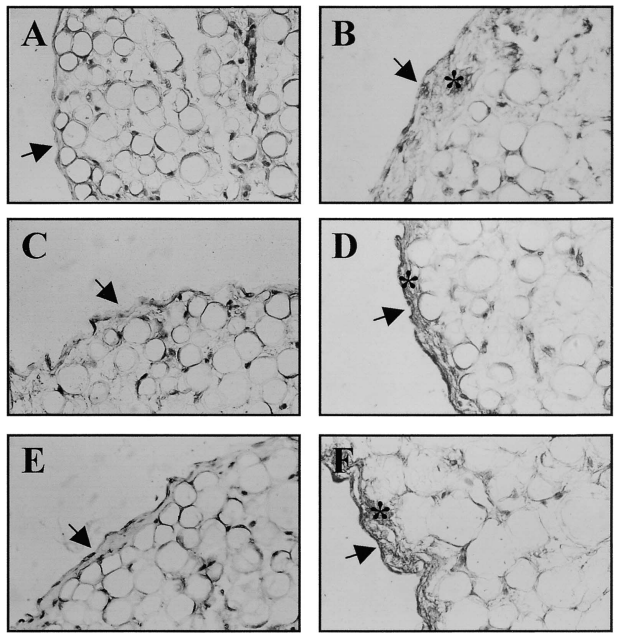

Fig. 1. Representative illustration comparing FN (*A* and *B*), Coll-I (*C* and *D*), and TGF-β1 (*E* and *F*) expression in peritoneal biopsy specimens from patients entering the PD program (renal failure controls) (A, C, and E) or patients on long-term PD (B, D, and F). Paraffin sections were subjected to cytochemical staining using the PAP method. Immunoreactive products were visualized using diaminobenzidine as chromagen and counterstained with hematoxylin. Constitutive expression of FN, Coll-I, and TGF-β1 was observed in peritoneal specimens from controls (A, C, and E, respectively). Peritoneal specimens from patients on long-term PD demonstrated enhanced FN, Coll-I, and TGF-β1 expression (B, D, and F, respectively), both within the mesothelium (➔) and in the submesothelium (✻). Abbreviations are: FN, fibronectin; Coll-I, collagen type I; TGF-β1, transforming growth factor-beta 1; PD, peritoneal dialysis; PAP, peroxidase-anti-peroxidase. Original magnification, ×400.

RESULTS

Expression of FN, Coll-I, and TGF-β1 in peritoneal biopsies

Cytochemical staining of peritoneal biopsy specimens demonstrated FN and Coll-I synthesis in mesothelial cells (Fig. 1). Increased expression of both FN and Coll-I was noted in chronic PD patients (Fig. 1), compared to those at the time of initiation of PD (Fig. 1), and was accompanied by increased TGF-β1 expression (Fig. 1).

The effects of high glucose concentration and emodin on HPMC proliferation, expression of PKCα, synthesis of TGF-β1, FN, and Coll-I, and the mechanisms leading to these changes, were investigated using an in vitro model whereby HPMC were preconditioned in either physiologic (5 mmol) or elevated (30 mmol) D-glucose concentrations for up to 1 week in the presence or absence of emodin. Optimal dose of emodin used in all experiments was 20 μg/mL (IC_{50} = 71.4 ± 43.6 μg/mL) (Figure 2 A to C). Emodin at this dose did not affect HPMC thymidine uptake or cell morphology (Fig. 2 A

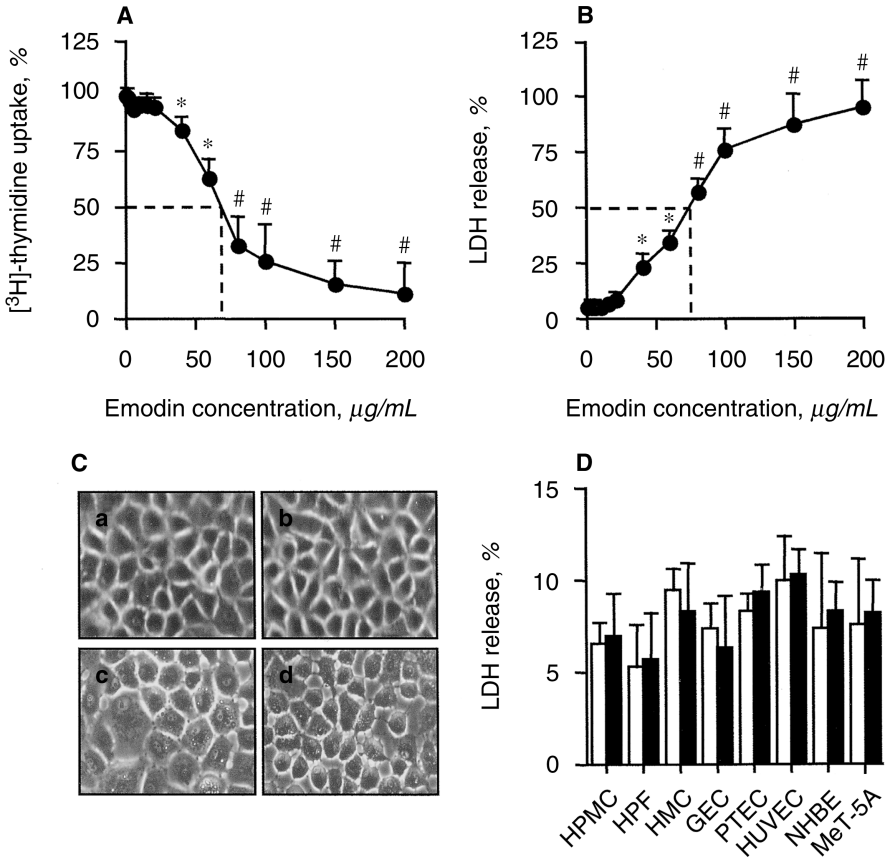

Fig. 2. The effects of emodin on HPMC proliferation (*A*) and membrane integrity (*B*) as determined by [^{3}H]-thymidine incorporation and LDH release respectively after incubation for 24 hours. (*C*) Morphology of HPMC with or without emodin (0, 20, 50, and 100 μg/mL) was assessed under phase contrast microscopy, respectively; original magnification, ×200). Emodin at 20 μg/mL did not affect HPMC proliferation, viability, or morphology. (*D*) Furthermore, emodin at this dose was not cytotoxic to fibroblasts, mesangial cells, epithelial, or endothelial cells, as demonstrated by insignificant changes in LDH release in the presence (■) or absence (□) of emodin. Abbreviations are: HPMC, human peritoneal mesothelial cells; LDH, lactate dehydrogenase. Data represented are mean ± SD of four individual experiments. *$P < 0.001$ emodin vs. control (no emodin); #$P < 0.0001$ emodin vs. control.

and C). In addition, it was not cytotoxic to HPMC cultured under physiologic (Fig. 2B) or elevated glucose concentrations, as well as a wide variety of other cell types, including HPF, HMC, GEC, PTEC, HUVEC, NHBE, and MeT-5A (Fig. 2D).

Effect of emodin on HPMC proliferation

HPMC proliferation was significantly reduced after incubation with 30 mmol D-glucose for 48 hours, compared to cells incubated with 5 mmol D-glucose (direct cell count, 43,200 ± 4100 vs. 31,200 ± 3600 cells, 5 mmol vs. 30 mmol D-glucose, $P < 0.001$). After 7 days, cells exposed to 30 mmol D-glucose exhibited 14.28% reduction in proliferation compared to control cells. Administration of emodin to cells cultured under 5 mmol and 30 mmol D-glucose reduced cell proliferation for both groups transiently (Fig. 3 A and B), resulting in an initial lag by 2.3 ± 0.3 days and 2.7 ± 0.7 days for 5 mmol and 30 mmol D-glucose, respectively. Thereafter, cell proliferation proceeded at the same rate as cells not exposed to emodin, and they reached confluency 2.4 ± 1.3 days later than control cells. Cell morphology of confluent cells remained unaltered in the presence or absence of emodin (Fig. 3C). Unless otherwise stated, all data were normalized to μg cellular protein.

Effect of emodin on tyrosine kinase and PKCα expression in HPMC under high glucose concentration

Previous studies have demonstrated in human bronchial epithelial cells that emodin is a tyrosine kinase inhibitor [28]. Our results also showed that emodin inhibited the activity of pure tyrosine kinase (EGFR) (Fig. 4). Elevated glucose concentration induced tyrosine kinase activity in HPMC, and emodin abrogated this induction by high glucose concentration (Fig. 4).

Since induction of PKCα plays a pivotal role in mediating the stimulatory effect of glucose on the synthesis of TGF-β1 and matrix proteins in various cells, we examined PKCα expression and the effects of emodin on PKCα activation in our in vitro model. Western blot analysis showed that 30 mmol D-glucose significantly increased PKCα expression in the cytosolic and membrane fractions in a time-dependent manner, commencing 12 hours after stimulation (data not shown). Maximum increase in PKCα expression was observed 48 hours after stimulation, in which cytosolic and membrane fractions increased 1.4-fold and 3.7-fold, respectively, compared to 5 mmol D-glucose (Fig. 5A, Table 1). While the addition of emodin to 5 mmol D-glucose had no effect on PKCα expression in either the cytosolic or membrane fraction (Fig. 5A, lanes 1 and 2; 0.515 ± 0.142

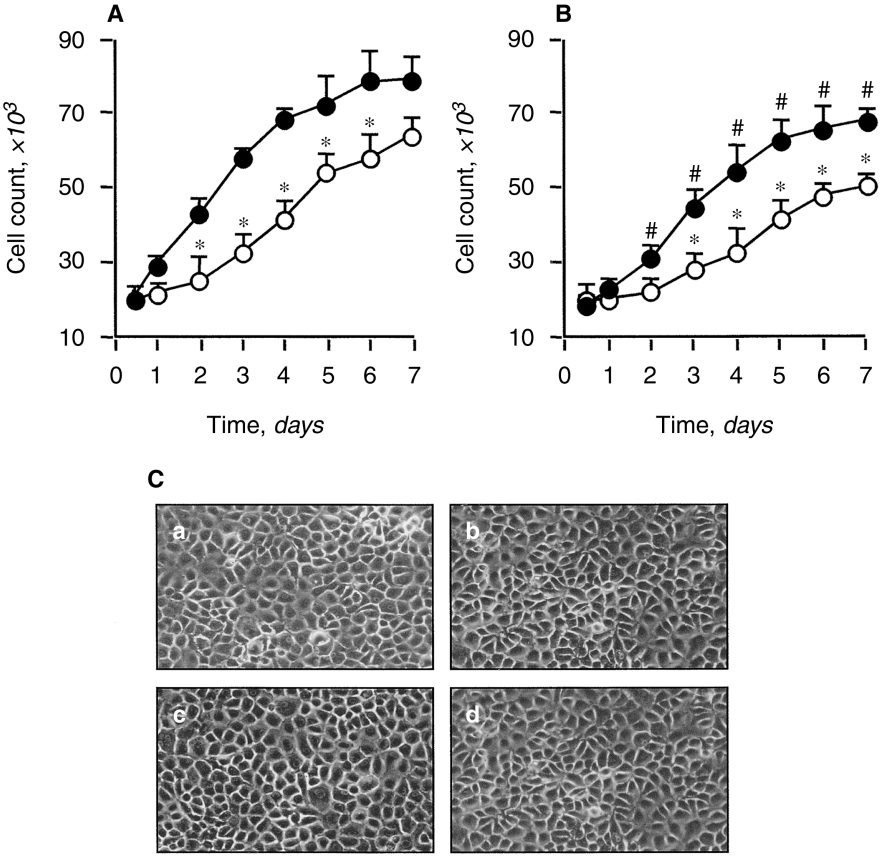

Fig. 3. Proliferation of HPMC as assessed by direct cell counting, under 5 mmol (physiologic) (*A*) or 30 mmol D-glucose (*B*), in the presence (○) or absence (●) of emodin. Proliferation was significantly reduced in HPMC cultured under 30 mmol D-glucose after 48 hours, compared to cells cultured in 5 mmol D-glucose. Addition of emodin delayed cell proliferation by 48 to 72 hours. On reaching confluency, no change was observed in cell morphology (C) in the presence (lower panels) or absence (upper panels) of emodin under 5 mmol (left panels) or 30 mmol (right panels) D-glucose. HPMC is human peritoneal mesothelial cell. $*P <$ 0.001, glucose alone vs. glucose and emodin at the same time point; $\#P < 0.001$ 5 mmol vs. 30 mmol D-glucose at the same time point, original magnification, ×200. Data represented are mean ± SD of six individual experiments.

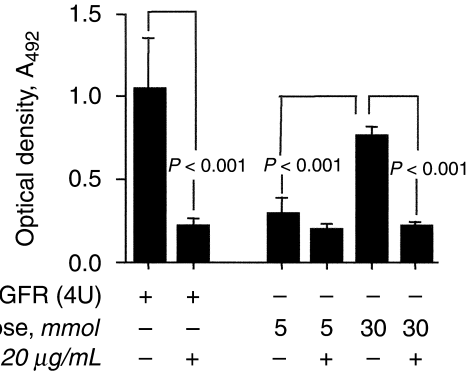

Fig. 4. Inhibition of tyrosine kinase activity by emodin. Cell lysates obtained under control or experimental conditions after 48 hours of stimulation were analyzed for tyrosine kinase activity as described. Purified tyrosine kinase (EGFR, 4U) was used as the positive control. Results are expressed as mean ± SD of three separate experiments.

DU vs. 0.415 ± 0.095 DU, $P = 0.504$; and 0.370 ± 0.015 DU vs. 0.331 ± 0.015 DU, $P = 0.487$, respectively), emodin lowered PKCα expression in HPMC exposed to 30 mmol D-glucose, resulting in levels similar to baseline (Fig. 5A, 0.503 ± 0.035 DU and 0.669 ± 0.048 DU for cytosolic and membrane fraction, respectively, $P < 0.05$ for both). To confirm the inhibitory effect of emodin on PKCα, PKC activation was achieved by exposing HPMC to PMA for 48 hours (Fig. 5A, lane 6; 0.737 ± 0.154 DU and 1.718 ± 0.214 DU for cytosolic and membrane fraction, respectively, $P < 0.05$, compared to HPMC not exposed to PMA); the concomitant presence of emodin and PMA returned PKCα activity to basal levels (Fig. 5A, lane 5; 0.588 ± 0.156 DU and 0.437 ± 0.057 DU, $P < 0.05$, compared to lane 6). Immunohistochemical analysis confirmed the increase in PKCα expression and its translocation to the cell membrane under elevated glucose concentration (Fig. 5B), and the abrogation of these changes by emodin (Fig. 5B).

Effect of glucose, PMA, and emodin on TGF-β1 synthesis by HPMC

Immunohistochemical analysis showed enhanced TGF-β1 synthesis in HPMC cultured under 30 mmol D-glucose with predominant perinuclear staining, compared to cells cultured under 5 mmol D-glucose (Fig. 6A). While emodin has no effect on constitutive TGF-β1 expression (Fig. 6A), concomitant addition of emodin ameliorated the induction of TGF-β1 by 30 mmol D-glucose, so that both the intensity and localization of TGF-β1 staining were similar to control (Fig. 6A).

The secretion of TGF-β1 by HPMC was determined

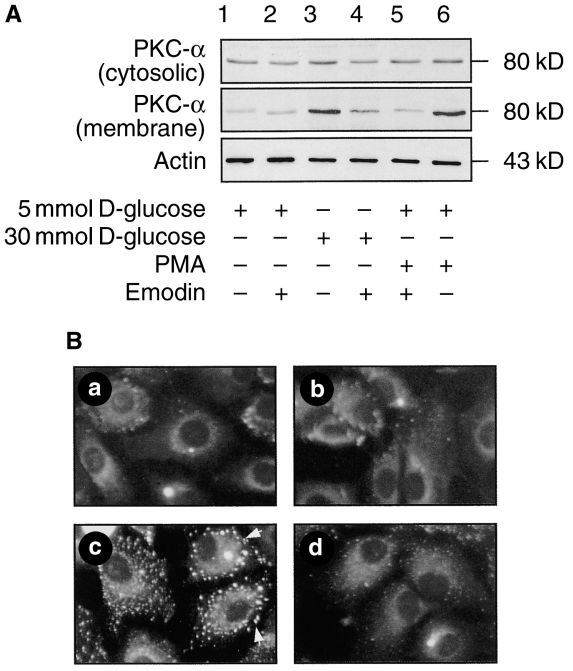

Fig. 5. Western blot analysis of PKCα expression in HPMC. (*A*) Cytosolic and membrane fractions were purified from 48 hour–stimulated HPMC as described in **Methods**, 10 μg total protein were subjected to SDS-PAGE and probed with PKCα antibody. Activation of PKC-α was associated with translocation to the cell membrane (lanes 3 and 6). Emodin did not alter PKCα expression under 5 mmol D-glucose, but ameliorated the increase in PKCα expression in the membrane fraction induced by 30 mmol D-glucose or PMA. One representative blot of four is shown. (*B*) Immunohistochemical analysis of HPMC for PKCα under (a) 5 mmol D-glucose, (b) 5 mmol D-glucose and emodin, (c) 30 mmol D-glucose, or (d) 30 mmol D-glucose and emodin. Cells were cultured under control or experimental conditions for 48 hours prior to fixation with cold acetone/methanol and were subsequently probed with specific antibody to PKCα (arrow). D-glucose (30 mmol) induced membrane translocation of PKCα (depicted by arrow). Emodin suppressed 30 mmol D-glucose–induced PKCα expression to basal levels, but did not affect constitutive PKCα expression. Original magnification, ×600. Abbreviations are: PKCα, protein kinase C α; HPMC, human peritoneal mesothelial cells; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMA, phorbol 12-myristate 13-acetate.

Table 1. Densitometric intensity of cytosolic and cell membrane–associated PKC-α under control and experimental conditions

	Densitometric intensity of PKC-α corrected to actin intensity *arbitrary units*		
	Total PKC-α	Cytosolic PKC-α	Membrane PKC-α
5 mmol D-glucose	0.885 ± 0.147	0.515 ± 0.142	0.370 ± 0.015
5 mmol D-glucose + emodin	0.746 ± 0.144	0.415 ± 0.095	0.331 ± 0.015
30 mmol D-glucose	2.104 ± 0.241[a]	0.734 ± 0.157[a]	1.370 ± 0.297[a]
30 mmol D-glucose + emodin	1.172 ± 0.214[b]	0.503 ± 0.035[b]	0.669 ± 0.048[b]
PMA	2.455 ± 0.187[a]	0.737 ± 0.154[a]	1.718 ± 0.214[a]
PMA + emodin	1.025 ± 0.298[b]	0.588 ± 0.156[b]	0.437 ± 0.057[b]

Abbreviations are: PKC-α, protein kinase C-α; PMA, phorbol 12-myristate 13-acetate. All samples stimulated for 48 hours were normalized to the "housekeeping" protein actin. Results are expressed as mean ± SD of four separate experiments.

[a] $P < 0.05$ when experimental conditions compared with 5 mmol D-glucose

[b] $P < 0.05$ when comparing results in the presence or absence of emodin

using a specific TGF-β1 ELISA, which demonstrated that the majority of TGF-β1 (>95%) in culture supernatants under control or experimental conditions was detected only after acid-activation, implying that it was synthesized mainly in its latent form. TGF-β1 synthesized by cells cultured in 5 mmol or 30 mmol D-glucose showed a time-dependent increase (Fig. 6B). Exposure of HPMC to 30 mmol D-glucose resulted in significantly higher supernatant concentrations of "latent" TGF-β1 at 24 hours and 48 hours compared to cells cultured under physiologic glucose concentration (3.72 ± 0.29 pg/μg cellular protein vs. 2.13 ± 0.23 pg/μg cellular protein at 24 hours, $P < 0.001$; and 4.30 ± 0.50 pg/μg cellular protein vs. 2.65 ± 0.32 pg/μg cellular protein at 48 hours, $P < 0.001$). Exposure of HPMC to 30 mmol mannitol or L-glucose did not alter TGF-β1 synthesis significantly compared to 5 mmol D-glucose (data not shown). Emodin did not affect the constitutive secretion of TGF-β1 under 5 mmol D-glucose, but it abrogated the induction by 30 mmol D-glucose (2.25 ± 0.15 pg/μg cellular protein at 24 hours, $P < 0.001$; 2.96 ± 0.33 pg/μg cellular protein at 48 hours, $P < 0.001$) (Fig. 6B). PMA markedly increased TGF-β1 secretion compared to 5 mmol D-glucose (6.92 ± 2.98 pg/μg cellular protein vs. 2.21 ± 0.21 pg/μg cellular protein, $P < 0.0001$) (Fig. 6C), but such increase was completely abrogated by emodin (2.58 ± 0.21 pg/μg cellular protein, $P < 0.0001$). Prior incubation of HPMC with neutralizing antibody to TGF-β1 (1 μg/mL) resulted in an 81.4% reduction of PMA-induced TGF-β1 secretion (3.41 ± 0.31 pg/μg cellular protein, $P < 0.0001$). No further change was observed with higher concentrations of neutralizing antibody to TGF-β1 (data not shown). Concomitant exposure of HPMC to PMA, neutralizing antibody to TGF-β1 and emodin resulted in supernatant TGF-β1 levels similar to those when HPMC was exposed to 5 mmol D-glucose alone.

Three to five percent of the total TGF-β1 secreted by HPMC under control or experimental conditions was in the "active" form and was detectable by ELISA without prior acid activation. Their bioactivity was confirmed using the mink lung epithelial cell growth inhibition assay (Table 2). This bioactive fraction increased 3.60- and 4.12-fold when the cells were cultured in 30 mmol D-glucose for 24 hours and 48 hours, respectively (Table 2). Mannitol or L-glucose (30 mmol) showed no effect (data not shown). In comparison, stimulation of HPMC with PMA for 24 hours and 48 hours induced bioactive TGF-β1 by 6.69- and 7.50-fold, respectively, compared to cells exposed to 5 mmol D-glucose. Emodin ameliorated the increase in bioactive TGF-β1 induced by elevated glucose or PMA (Table 2). To ascertain whether residual

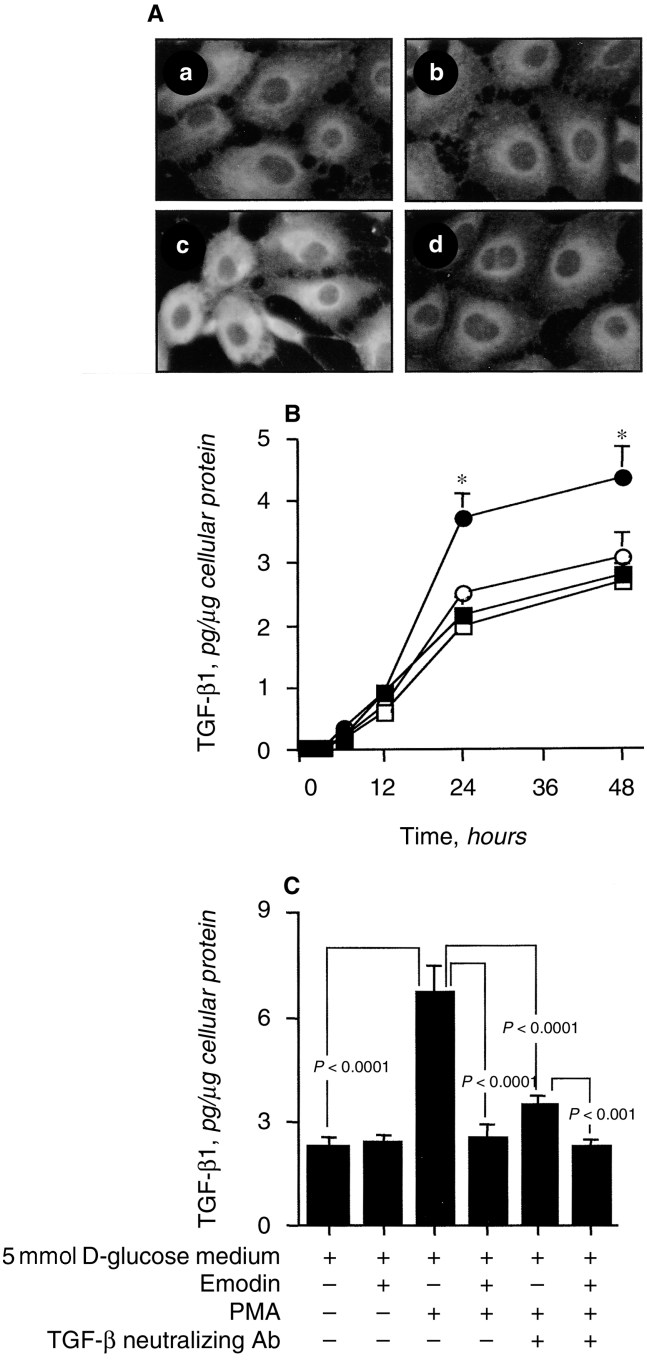

Fig. 6. (A) Immunohistochemical analysis of HPMC for TGF-β1 expression under (a) 5 mmol D-glucose, (b) 5 m D-glucose and emodin, (c) 30 mmol D-glucose, or (d) 30 mmol D-glucose and emodin. Cells were cultured under control or experimental conditions for 48 hours prior to fixation with cold acetone/methanol and were subsequently probed with specific antibody to TGF-β1. Emodin suppressed 30 mmol D-glucose–induced TGF-β1 expression to basal levels, but did not affect constitutive TGF-β1 expression. Original magnification, ×600. (*B*) Effect of glucose and emodin on TGF-β1 secretion by HPMC. HPMC were cultured in 5 mmol D-glucose in the presence (□) or absence (■) of emodin, or in 30 mmol D-glucose with (○) or without (●) emodin for time periods up to 48 hours. TGF-β1 content was measured in the supernatant ($N = 4$). Emodin had no effect on TGF-β1 secretion by HPMC cultured under 5 mmol (physiologic) D-glucose, but abrogated the increase in TGF-β1 secretion induced by 30 mmol D-glucose to levels similar to cells exposed to physiologic glucose concentration. $^*P < 0.001$, 30 mmol D-glucose vs. 5 mmol D-glucose at T = 24 and 48 hours. (*C*) Effects of PMA-stimulated PKC activation, emodin, and neutralizing TGF-β1 antibody on TGF-β1 secretion by HPMC. HPMC were stimulated with PMA (100 nmol) in the presence or absence of emodin or TGF-β1 neutralizing antibody for 48 hours. TGF-β1 secretion was assessed in the supernatant ($N = 4$). Emodin suppressed PMA-stimulated TGF-β1 secretion to basal levels, and its suppressive effect was more pronounced compared to TGF-β1 neutralizing antibody. Abbreviations are: HPMC, human peritoneal mesothelial cells; TGF-β1, transforming growth factor-β1; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C.

Table 2. Effect of D-glucose, PMA, and emodin on TGF-β1 bioactivity

	TGF-β1 bioactivity pg/μg cellular protein	
	T = 24 h	T = 48 h
5 mmol D-glucose	0.068 ± 0.01	0.072 ± 0.01
5 mmol D-glucose + emodin	0.065 ± 0.02	0.075 ± 0.02
30 mmol D-glucose	0.245 ± 0.06[a]	0.297 ± 0.03[a]
30 mmol D-glucose + emodin	0.063 ± 0.01[b]	0.079 ± 0.02[b]
PMA	0.455 ± 0.09[a]	0.541 ± 0.12[a]
PMA + emodin	0.066 ± 0.04[b]	0.089 ± 0.07[b]

PMA is phorbol 12-myristate 13-acetate. Results are expressed as mean ± SD of four separate experiments.
[a] $P < 0.05$ comparing experimental conditions with 5 mmol D-glucose
[b] $P < 0.05$ comparing results in the presence or absence of emodin

PMA ± emodin remaining in the conditioned media after incubation with HPMC could induce MLEC TGF-β1 synthesis, and thus alter MLEC growth, TGF-β1 levels in the conditioned media were determined using ELISA before and after administration to MLEC. Our results showed that the residual PMA ± emodin had no significant effect on MLEC TGF-β1 synthesis or cell growth (data not shown).

Effect of glucose, PMA, and emodin on FN and Coll-I synthesis by HPMC

Using "in-house" FN and Coll-I ELISA, we observed up-regulation of FN synthesis and secretion after stimulation of HPMC with 30 mmol D-glucose for 48 hours, compared to cells cultured under 5 mmol D-glucose (36.3 ± 3.6 vs. 157.8 ± 9.1 ng/μg cellular protein for 5 mmol and 30 mmol D-glucose, respectively, for secreted FN; 19.4 ± 5.2 vs. 79.4 ± 12.2 ng/μg cellular protein for 5 mmol and 30 mmol D-glucose, respectively, for cell-associated FN, $P < 0.05$ for both) (Table 3). FN synthesis was also significantly increased upon PKC activation (217.0 ± 21.8 and 91.5 ± 15.3 ng/μg cellular protein for secreted and cell-associated FN, respectively, $P < 0.05$ compared to control). Emodin had no effect on FN synthesis under physiologic glucose concentration, but ameliorated both glucose-induced and PMA-stimulated FN synthesis. The inhibitory effect of emodin was similar to TGF-β1 neu-

Table 3. Determination of secreted and cell-associated FN and Coll I in HPMC under control and experimental conditions

	Concentration *ng/μg cellular protein*		
	Secreted FN	Cell-associated FN	Cell-associated Coll-I
5 mmol D-glucose	36.3 ± 3.6	19.4 ± 5.2	16.8 ± 8.1
5 mmol D-glucose + emodin	34.6 ± 3.9	19.7 ± 4.3	17.8 ± 4.6
5 mmol D-glucose + normal IgG	35.0 ± 4.8	18.9 ± 6.2	17.2 ± 2.1
5 mmol D-glucose + TGF-β1 Ab	37.7 ± 2.6	19.2 ± 4.7	16.5 ± 5.1
30 mmol D-glucose	157.8 ± 9.1[a]	79.4 ± 12.2[a]	56.3 ± 12.5[a]
30 mmol D-glucose + emodin	39.8 ± 5.4[b]	22.4 ± 5.3[b]	18.4 ± 4.6[b]
30 mmol D-glucose + normal IgG	159.3 ± 10.5[a]	77.2 ± 10.5[a]	57.1 ± 8.3[a]
30 mmol D-glucose + TGF-β1 Ab	43.7 ± 8.2[b]	25.5 ± 8.1[b]	20.4 ± 5.9[b]
PMA 100 nmol	217 ± 21.8[a]	91.5 ± 15.3[a]	70.4 ± 10.2[a]
PMA + emodin	47.1 ± 9.2[b]	26.5 ± 7.3[b]	19.4 ± 7.5[b]
PMA + normal IgG	210.3 ± 19.7[a]	89.6 ± 11.7[a]	67.9 ± 8.1[a]
PMA + TGF-β1 Ab	54.2 ± 10.5[a,b]	32.9 ± 7.5[a,b]	28.5 ± 3.1[b]

Abbreviations are: FN, fibronectin; PMA, phorbol 12-myristate 13-acetate; IgG, immunoglobulin G; TGF-β1 Ab, transforming growth factor-beta 1 antibody. Enzyme-linked immunosorbent assay (ELISA) was used to analyze cell supernatants and lysates for FN and Coll-I obtained 48 hours of experimental stimulation. Results are expressed as mean ± SD of four separate experiments.

[a] $P < 0.05$ comparing experimental conditions with 5 mmol D-glucose

[b] $P < 0.05$ comparing 30 mmol D-glucose or PMA in the presence or absence of emodin or TGF-β1 Ab

tralizing antibody, and was more marked with elevated glucose concentration than PMA (Table 3).

Elevated glucose concentration and PMA also modulated the translation of Coll-I. Coll-I was not constitutively secreted from HPMC cultured under 5 mmol D-glucose, nor was Coll-I secretion altered by high glucose concentration or PMA stimulation (data not shown). D-glucose (30 mmol) induced the expression of only the cell-associated Coll-I, compared to 5 mmol D-glucose (56.3 ± 12.5 vs. 16.8 ± 8.1 ng/μg cellular protein, $P < 0.05$), and such stimulation was abrogated by emodin. Exposure of HPMC to PMA markedly increased cell-associated Coll-I (70.4 ± 10.2, $P < 0.05$ compared to control), which was again ameliorated by emodin and anti-TGF-β1 antibody (Table 3). Western blot analysis corroborated the results on FN and Coll-I by ELISA (Fig. 7 A and B).

Localization of FN and Coll-I in HPMC cultured under control or experimental conditions for periods up to 1 week was assessed using immunohistochemical staining (Fig. 7C). Significantly increased intracellular FN and Coll-I was observed after 48 hours of 30 mmol D-glucose stimulation (data not shown), while induction of FN and Coll-I deposition into the extracellular milieu was observed at 72 hours, and the latter progressed to form an extensive matrix by 1 week (Fig. 7C). Emodin reduced the increased matrix synthesis induced by high glucose concentration to near basal levels, and the ameliorating effect was evident even in HPMC exposed to 30 mmol D-glucose for 1 week. Despite the reduction in matrix synthesis by emodin, cell attachment was unaffected, as determined by cell counting (62,000 ± 967 cells/cm^2, 62,630 ± 1010 cells/cm^2, 61,567 ± 1009 cells/cm^2, and 61,789 ± 1034 cells/cm^2 for HPMC cultured under 5 mmol D-glucose, 5 mmol D-glucose with emodin, 30 mmol D-glucose, and 30 mmol D-glucose with emodin, respectively; $P = 0.70$, 0.69, and 0.78, respectively, when compared to 5 mmol D-glucose alone after 1 week of culture under control or experimental conditions). Control experiments using 30 mmol mannitol or L-glucose did not show any significant change in FN or Coll-I expression compared to 5 mmol D-glucose (data not shown).

Effect of glucose and emodin on CREB phosphorylation

CRE of the FN gene has been demonstrated to mediate increased FN synthesis in mesangial cells induced by high glucose concentration or TGF-β1 [31, 32]. We therefore investigated CREB phosphorylation in nuclear extracts isolated from HPMC cultured under physiologic and elevated glucose concentrations using Western blot analysis. Anti-phosphoCREB (pCREB) antibody used in this study was raised against the phosphoserine in a portion of the CREB protein that shared 100% homology with the phosphoserine site of the CREB-related protein activated transcription factor-1 (ATF-1) [42]. Consequently, ATF-1 could also be detected by this antibody. Our results demonstrated that while total CREB content remained unaltered under control and experimental conditions, 30 mmol D-glucose increased CREB phosphorylation at serine 133 by 1.84-fold, compared to 5 mmol D-glucose (0.46 ± 0.25 DU vs. 0.25 ± 0.09 DU). This was accompanied by a 1.36-fold increase in ATF-1 at serine 63 (Fig. 8A, lanes 1 and 3). PMA also activated CREB and ATF-1 (Fig. 8A, lane 6; 0.57 ± 0.01 and 0.22 ± 0.01 DU, respectively). In comparison, emodin significantly reduced the induction of CREB and ATF-1 phosphorylation by both high glucose and PMA (Fig. 8A, lanes 4 and 5, respectively). In control experiments, neither 30 mmol mannitol nor L-glucose altered phosphorylation of CREB or ATF-1, as compared to 5 mmol D-glucose (data not shown).

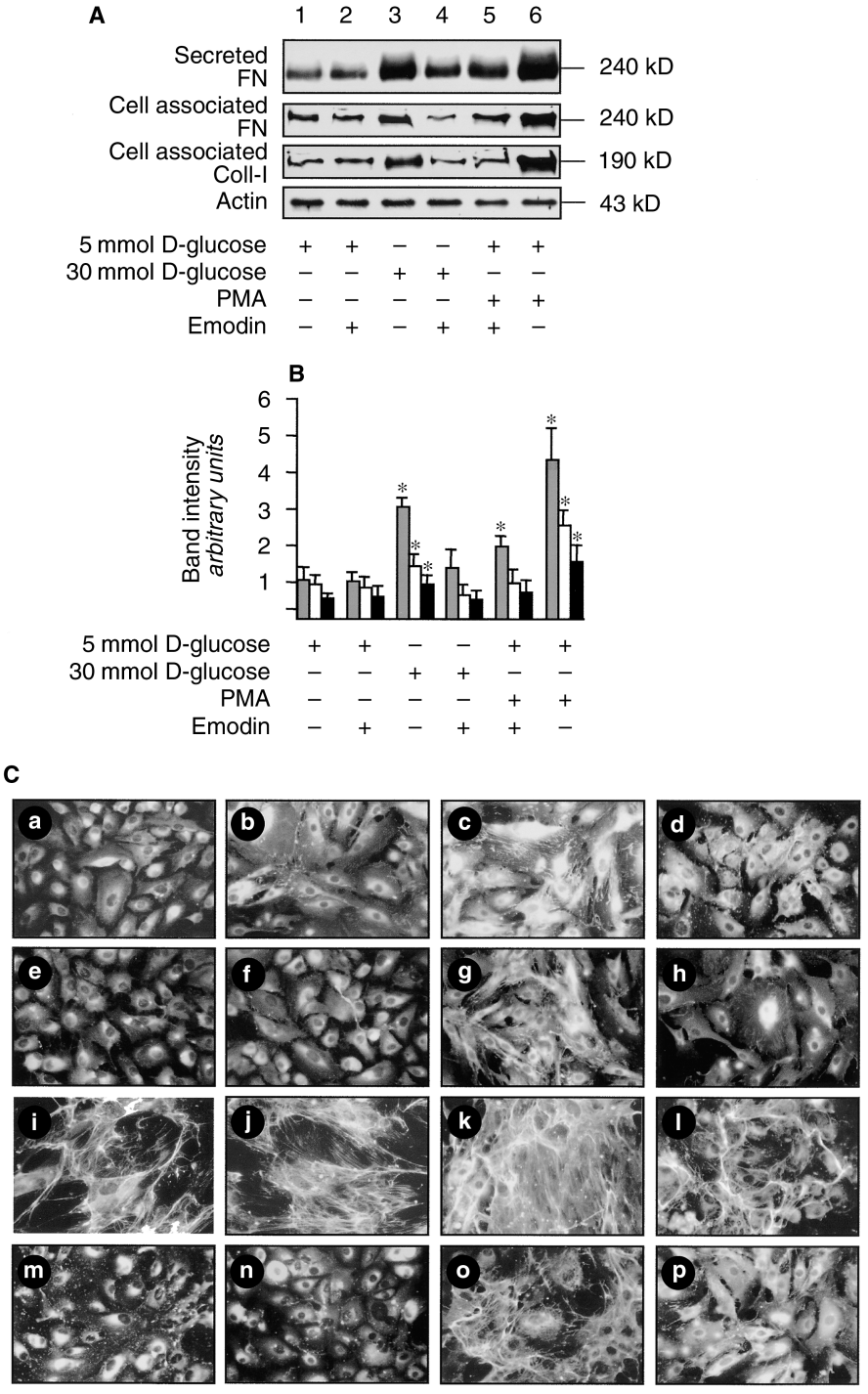

Fig. 7. Western blot analysis of FN and Coll-I synthesis by HPMC showing the effects of glucose, PMA, and emodin. (*A*) Culture supernatant or total cell lysate was extracted from HPMC after 48 hours of stimulation, subjected to SDS-PAGE, and probed with antibodies to FN or Coll-I. Emodin ameliorated the increase in FN (secreted and cell-associated) and Coll-I (cell-associated only) synthesis induced by 30 mmol D-glucose and PMA to control levels. One representative blot of four separate experiments is shown. (*B*) The intensity of the detected bands of secreted FN (▨), cell-associated FN (□), and cell-associated Coll-I (■) were analyzed by densitometric scan, normalized to actin, and expressed as arbitrary units. (*C*) Immunohistochemical analysis of HPMC for FN and Coll-I under control or experimental conditions. HPMC were cultured in 5 mmol D-glucose (a, e, i, m), 5 mmol D-glucose and emodin (b, f, j, n), 30 mmol D-glucose (c, g, k, o), or 30 mmol D-glucose and emodin (d, h, l, p) for 72 hours (a through h) or one week (i through p) prior to fixation with cold acetone/methanol. Cells were subsequently stained for FN (a through d, i through l) or Coll-I (e through h, m through p). Cytoplasmic and extracellular deposition of FN, the latter in fibrillar form, was noted after exposure to 5 mmol D-glucose for up to one week (a vs. i), but was accelerated when cells were exposed to 30 mmol D-glucose (c and k). Fibrillar FN deposition was evident 72 hours after exposure to elevated glucose concentration (c). D-glucose (30 mmol) also induced Coll-I synthesis in a time-dependent manner similar to FN (e, g, m, o). Addition of emodin to 30 mmol D-glucose for 72 hours reduced cytoplasmic and fibrillar FN, as well as Coll-I expression, although perinuclear staining of FN and Coll-I was still evident (c and d, g and h, respectively). An extensive matrix was deposited by cells cultured in 30 mmol D-glucose for one week, containing predominantly FN (k) and to a lesser extent Coll-I (o). Both were suppressed to near basal levels when emodin was present concomitantly (l and p). Original magnification, ×400. Abbreviations are: FN, fibronectin; Coll-I, collagen type I; HPMC, human peritoneal mesothelial cells; PMA, phorbol 12-myristate 13-acetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

DISCUSSION

Mesothelial cells line the peritoneal cavity and constitute a protective bioactive barrier that plays a key role in maintaining the structural and functional integrity of the peritoneal membrane [1]. In addition, the normal synthesis of peritoneal membrane constituents by peritoneal mesothelial cells is essential for peritoneal preservation. Surgical, chemical, or infective injury to the mesothelium initiates pathologic processes that culminate in disruptions of both structural and functional integrity of the peritoneal membrane [2, 10–12]. Conventional peritoneal dialysis solutions contain glucose at high concentrations as the osmotic agent. Continuous exposure of the peritoneum to these unphysiologic solutions is associated with abnormalities in cellular proliferation, synthesis of cytokines and growth factors, and increased

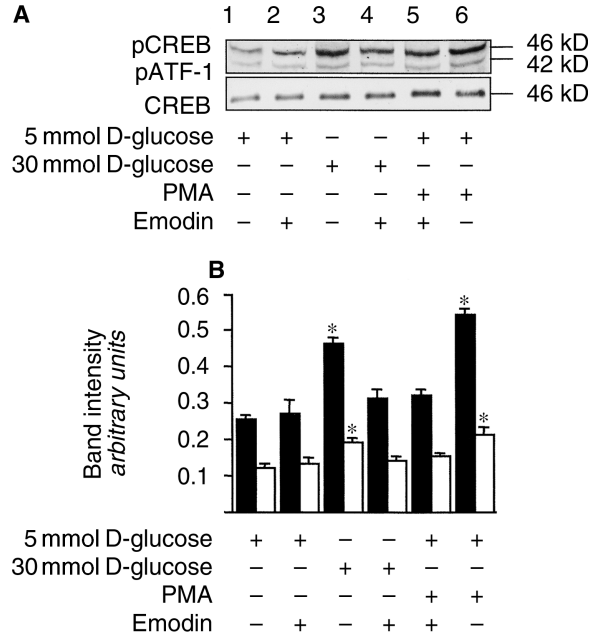

Fig. 8. Western blot analysis showing the effects of glucose, PMA, and emodin on CREB and ATF-1 phosphorylation by HPMC after 48 hours of stimulation. (*A*) Nuclear extracts were subjected to SDS-PAGE and probed with antisera to phosphoCREB and phosphoATF-1. While no significant difference was observed for total CREB expression under different experimental conditions, emodin reduced 30 mmol D-glucose- or PMA-induced CREB and ATF-1 phosphorylation, but had no effect on the constitutive activation of the transcription factors. One representative blot of three separate experiments is shown. (*B*) The intensity of the detected bands of phosphoCREB (■) and phosphoATF-1 (□) were analyzed by densitometric scan, normalized to CREB, and expressed as arbitrary units. Abbreviations are: PMA, phorbol 12-myristate 13-acetate; CREB, cAMP-responsive element binding protein; ATF-1, activating transcription factor-1.

synthesis of matrix components by the mesothelial cells and deposition of matrix within the submesothelium [3, 9, 13–15]. Increased matrix deposition is precedent to peritoneal fibrosis and a major contributing factor to failure of peritoneal transport functions. In line with published work, we have demonstrated accumulation and deposition of FN and Coll-I within peritoneal biopsies obtained from chronic PD patients accompanied by enhanced TGF-β1 expression, compared to biopsies obtained from new PD patients. While previous studies have implicated prolonged exposure of the peritoneum to glucose at high concentrations as a major contributing factor for these profibrotic processes, there is as yet no effective means to ameliorate or prevent the detrimental effects of concentrated glucose on peritoneal mesothelial cells.

HPMC cultured in vitro provide a relevant in vitro model to study the effect of PD solutions on cell proliferation and function, since previous in vitro studies have demonstrated that they possess the same immunohistochemical markers as peritoneal mesothelial stem cells [43]. In addition to the suppressive effect of high glucose concentration on HPMC proliferation [13], in this study we demonstrated for the first time that high ambient glucose concentration induced PKCα autocrine phosphorylation of CREB and ATF-1 in HPMC, and induced TGF-β1 mediated fibrillar and cytoplasmic FN and Coll-I synthesis. Activation of PKCα, exemplified by its translocation from the cytosol to the cell membrane in HPMC by concentrated glucose, also up-regulated FN and TGF-β1 gene transcription [30], but not that of Coll-I (unpublished data). Our results also showed that the synthesis of laminin and collagen types III and IV was similarly induced by concentrated glucose (unpublished data). These effects were a result of elevated D-glucose concentration, per se, rather than hyperosmolality, since there was no significant effect with concentrated mannitol or L-glucose compared to physiologic control. Similar effects of concentrated glucose have been reported in human mesangial cells, which have also been related to PKC activation [44–46]. It has been suggested that glucose metabolism, either through the pentose phosphate shunt or through glycerolipid production, leads to an increase in diacylglycerol and, subsequently, activation of PKC. In this context, Ha et al [13] have demonstrated that high glucose–induced diacylglycerol and PKC up-regulation mediated TGF-β1 and FN synthesis by peritoneal mesothelial cells.

Activation of PKC has been shown to modulate transcription factors, for example, c-fos and c-jun, through the activated protein-1 (AP-1) binding site. Promotor regions of TGF-β1, FN, and Coll-I have also been demonstrated to contain the AP-1 binding consensus sequence, which may therefore represent one mechanism by which high ambient glucose concentration up-regulates TGF-β1 and matrix synthesis [47, 48]. On the other hand, it has been reported that PKC activation and CREB phosphorylation are involved in elevated glucose–mediated induction of FN transcription in human mesangial cells, mediated through a consensus CRE located at −170 bp of the FN gene [31, 32]. Our results in the present study showed that high glucose–induced FN synthesis was also associated with phosphorylation of CREB in HPMC. Phosphorylation of CREB at serine 133 appears to be essential for its activation, and can occur through numerous signaling pathways, for example, PKA stimulation, adenyl cyclase pathway, p42/44 MAP kinase, p38 kinase, or induction of intracellular Ca^{2+} [49–51]. Unlike mesangial cells [32, 52], and in concordance with a previous study on FN synthesis [13], we have found that concentrated glucose was able to induce FN and Coll-I synthesis in HPMC in the absence of exogenous TGF-β1 or fetal calf serum (FCS). These results suggest that concentrated D-glucose per se plays a pivotal role in the induction of matrix synthesis in HPMC.

Other investigators have reported that emodin may possess antiproliferative and/or anti-inflammatory activities in mesangial cells, bronchial epithelial cells, lung squamous

cell carcinoma cell line, and T lymphocytes, at concentrations that vary from 1.35 μg/mL (5 μmol) to 100 μg/mL (370 μmol) [25, 26, 28]. The observed variability in the cellular effects of emodin may be attributed to species differences in its phytologic origin, or heterogeneity of response in different cell types. To address these issues, we have used highly purified emodin from a single batch and have examined its effects on cell proliferation and viability in different cell types. A prerequisite to further experiments in vivo is that the emodin species being tested must not adversely affect cell viability. Our data showed that emodin at 20 μg/mL (74 μmol) was nontoxic to HPMC exposed to either physiologic or elevated D-glucose concentration, as evidenced by assessment of cell proliferation and LDH release. We have, therefore, used this dose in subsequent experiments. Furthermore, our emodin at this concentration did not induce membrane damage in a wide variety of cell types, including peritoneal fibroblasts and other cells that may be involved in matrix synthesis or tissue fibrosis. It is of interest to note that emodin at 20 μg/mL did not affect cell proliferation in 80% confluent HPMC, yet proliferation was transiently inhibited in newly seeded cells cultured under control and experimental conditions. This differential effect may suggest that subconfluent cells could be more sensitive to the effects of emodin. The antimitogenic effect of emodin in relation to cell density is currently being studied. A major finding in this study was that emodin consistently suppressed concentrated glucose-induced TGF-β1 bioactivity, FN, and Coll-I synthesis in HPMC. These ameliorating effects of emodin on TGF-β1 and matrix synthesis were genuine and not related to differences in cell number, since our results showed similar cell numbers under control and experimental conditions at confluence when the experiments were performed. Nevertheless, the observed reduction in mesothelial cell proliferation in the presence of 30 mmol D-glucose or emodin suggested alterations in the dynamic processes of cell turnover, which could be of particular relevance in the regenerative response to injury. In animal models of diabetic nephropathy, reduction of mesangial proliferation by antimitogenic agents was accompanied by amelioration of glucose-induced matrix synthesis [53]. It remains to be investigated whether the ameliorating effect of emodin on D-glucose–induced matrix synthesis by HPMC might be causally related to its precedent antimitogenic effect that lasted for approximately 48 hours. It is noteworthy that, despite the initial inhibition of HPMC proliferation, emodin had no effect on the constitutive synthesis of TGF-β1 and matrix by HPMC under 5 mmol D-glucose.

Our data demonstrated that the abrogation of elevated D-glucose–induced TGF-β1 and matrix synthesis in HPMC by emodin was mediated through the inhibition of PKC activation. The results suggest a more pronounced inhibitory effect with emodin compared to neutralizing antibody to TGF-β1, since the addition of emodin resulted in the complete suppression of PMA-induced TGF-β1 and matrix synthesis. Since both soluble and cell-associated FN were decreased by emodin, it is likely that emodin suppressed FN synthesis, rather than inhibiting its accumulation and deposition within the extracellular milieu. Although we have demonstrated that PKCα activation plays an important role in D-glucose–induced matrix synthesis in HPMC, it remains possible that other PKC isoforms may be involved in the pathogenesis of peritoneal fibrosis, as has been reported in diabetic nephropathy [54]. In this regard, we are investigating the potential effects of emodin on other isoforms of PKC in the presence of different concentrations of D-glucose.

Emodin inhibits different protein kinases, including casein kinases 1 and 2, PKA, and tyrosine kinase [55, 56]. Whether emodin can inhibit other kinases, such as MAPK and c-jun NH_2-terminal kinase (JNK), is currently being investigated. The inhibitory action of emodin is dependent on its ability to penetrate into the active site of the enzymes [29]. Crystallography studies by Battistutta et al [29] demonstrated that emodin enters the nucleotide-binding site of casein kinase II in the proximity of the site normally occupied by the natural cosubstrate. This led to structural changes in the N-terminal lobe of the enzyme, resulting in the inability of casein kinase II to bind to its cosubstrate [29]. It is speculated that similar molecular mechanisms may explain the inhibitory action of emodin on PKC in HPMC.

CONCLUSION

We have demonstrated that emodin can effectively ameliorate the detrimental effects of concentrated D-glucose on HPMC, namely the induction of TGF-β1 bioactivity and matrix synthesis, through inhibition of PKCα activation and CREB phosphorylation, without compromising cellular viability. Further studies will need to be be undertaken in an animal model of peritoneal fibrosis to confirm our in vitro findings. Amelioration of TGF-β1 mediated tissue fibrosis using emodin may provide important clinical potential in the long-term preservation of structural and functional integrity of the peritoneal membrane in patients on PD.

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